مطالعه مولکولی گلوکز –6 فسفات دهیدروژناز مدیترانه ای در شمالغرب ایران

افشین فتحی [1]، مهدی ولی زاده [2]،حبیب عنصری [3]، روح اله مرادپور[4]، فیروز امانی [5]

1- استادیار گروه هماتولوژی و انکولوژی کودکان، دانشگاه علوم پزشکی اردبیل

2- گروه ژنتیک، کارشناس ارشد ژنتیک

3- استادیار گروه زیست شناسی سلولی و مولکولی، دانشگاه آزاد اسلامی واحد مرند

4- گروه ژنتیک، کارشناس ارشد ژنتیک

5- استادیار گروه آمار زیستی، دانشگاه علوم پزشکی اردبیل

نویسنده مسئول: استادیار گروه هماتولوژی و انکولوژی کودکان، دانشگاه علوم پزشکی اردبیل ، اردبیل، ایران

چکیده

نقص آنزیم گلوکز6- فسفات دهیدروژناز شایعترین نقص آنزیمی انسانی است که بیش از 400 میلیون نفردر سراسر جهان به آن مبتلا هستند این آنزیم مرحله اول در مسیر پنتوز فسفات را کاتالیز می کند که در این مرحله گلوکز 6-فسفات را به 6-فسفو گلوکونات تبدیل می کند و همراه با آن احیاء $NADP^+$ را منجر می شود . این مسیر یک منبع مهم تولید NADPH است . NADPH، با حفظ گلوتاتیون به شکل احیاء در حفاظت سلول در برابر تنش های اکسیداتیو نقش دارد.

هدف این مطالعه شناسایی مولکولی جهش مدیترانه ای گلوکز-6-فسفات دهیدروژنازدربیماران مبتلا درشمالغرب ایران بود. درمطالعه حاضراز 90 نمونه خون بیماران زن و مرد غیر خویشاوند DNA, توسط روش استخراج DNA ژنومی سریع (RGDE) استخراج شد. به منظورجستجوی جهش مدیترانه ای، روش های تعیین توالی و PCR-RFLP مورداستفاده قرارگرفتند. این مطالعه نشان داد که 61 نمونه از 90 نمونه (67.77%) جهش مدیترانه ای دارند. داده ها نشان میدهند که جهش G6PD مدیترانه ای شایعترین نوع جهش درشمالغرب ایران است.

واژهای کلیدی: گلوکز 6-فسفات دهیدروژناز، جهش مدیترانه ای ، شمالغرب ایران

Molecular investigation of Mediterranean glucose-6-phosphate dehydrogenase in North West of Iran

Fathi Afshin[1],Mehdi Valizadeh[2],Onsori Habib[3],Moradpour Rouhollah[4],Amani Firouz[5].

1- Pediatric Hematology & Oncology Department. Ardabil University of Medical Sciences, Ardabil, Iran

2- Genetics Department, M. A. in Genetics

3- Cellular and Molecular Biology Department,Marand Branch, Islamic Azad University, Marand, Iran

4-Genetics Department, M .A. in Genetics

5- Community Medicine Department, Ardabil University of Medical Science, Ardabil, Iran

Corresponding author: Pediatric Hematology & Oncology Department. Ardabil University of Medical Sciences, Ardabil, Iran

Abstract

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common human enzyme deficiency affecting more than 400 million people worldwide.This enzyme catalyse the first step in pentose phosphate pathway (conversion of glucose-6-phosphate to 6-phosphogluconat) with the concomitant reduction of $NADP^+$. This pathway is an important source of NADPH. By preserving and regenerating reduced form of glutathione, NADPH plays a major role in a cell's stability to withstand oxidative stress. The aim of this study was molecular identification of Mediterranean mutation in Glucose-6-phosphate dehydrogenase in affected patients in North West of Iran. In the present study,from 90 blood samples of unrelated male and female patients, DNA was extracted by Rapid Genomic DNA Extraction (RGDE) method.In order to search for Mediterranean mutation, PCR-RFLP and sequencing methods were used.This study, revealed that 61 samples out of 90 have the Mediterranean mutation (67.77%).the data indicate that the G6PD Mediterranean mutation is the most common in North West of Iran.

Keyword: G6PD,Mediterranean, mutation, North West of Iran.

Introduction

The housekeeping enzyme glucose-6-phosphate dehydrogenase (G6PD) catalyzes the first step in pentose phosphate pathway (conversion of glucose-6-phosphat to 6-phosphoglucono lactone) with the concomitant reduction of $NADP^+$. This pathway is an important source of NADPH that is essential for maintaining adequate intracellular level of reduced forms of glutathione and other sulphydryl groups. By preserving and regenerating reduced forms of glutathione as well as promoting the stability of catalase, NADPH plays a major role in the stability of cell to withstand oxidative stress. In red blood cells, since G6PD is the only source of NADPH, defense against oxidative damage is dependent on its activity [1-2]. The G6PD gene is located on the Xq28 region of X chromosome. It contains 13 exons and 12 introns and is 18.5 kb in length. The active enzyme is composed variably of two or four identical 515 amino acid subunits; each monomer has a molecular weight of 59 kDa. G6PD deficiency is the most common human enzymopathy and affects 400 million people worldwide. Although the majority of people with this disease are asymptomatic, some of the clinical symptoms associated with deficiency are acute hemolytic anemia in association with infection or following the ingestion of some drugs or fava beans (favism), neonatal jaundice and in severe deficiency, chronic non-spherocytic hemolytic anemia (CNSHA) [3-4].

Although Iranian population consists of different racial groups, but the overall incidence of G6PD deficiency in Iranian population is estimated around 10%-14.9% [5-6].

According to previous studies, the most prevalent mutations in Iran are Mediterranean, Chatham and Cosenza[6]. The aim of the present study is to investigate the frequency rate of the Mediterranean mutation at 563nt(C→T) in north-west of Iran.

Methodology

In this cross-sectional study,90 Peripheral blood samples(2-5 ml in 300 µEDTA 0.5 M) from unrelated patients with favism were collected from the hospitals of the north-west of Iran (including Ardebil, Tabriz and Urmia provinces). The blood samples collection process was performed with the ethics committee approval and the patients' informed consent through telephone correspondences. The falcons were held in the temperature of -20^{oC}until the DNA extraction. Qualitative measurement of the enzyme activity was performed using Fluorescent Spot Test and Saba laboratory kit. The basis of this method is the catalytic activity of G6PD enzyme in conversion of G6PD Glucose 6-phosphate to 6-Phosphogloconate and simultaneous revival of NADP to NADPH2. The produced NADPH2 has fluorescent specifications under UV (365nm). The fluorescence intensity in the blood of healthy individuals is positive (strong) and in the blood of patients with G6PD deficiency is low or negative.

DNA Extraction and Amplification

Genomic DNA was extracted from Peripheral blood leukocytes using Rapid Genomic DNA Extraction (RGDE) method [7].

All 90 DNA samples were analysed by PCR-RFLP method for the C to T mutation at nt

563, which is characteristic of G6PD Mediterranean.PCR reaction mixture containing 30 pmol of eachprimers (F-Med: 5' CCC CGA AGA GGA ATT CAA GGG GGT 3') as a forward and R-Med: 5' GAA GAG TAG CCC TCG AGG GTG ACT 3') as a reverse, 12/5µl DFS-Taqmaster mix (Bioron-Germany) and about 1µg genomic DNA on a thermal cycler (SensoQuest-Germany).PCR conditions were as follows: Initial denaturation of DNA at 95 ˚C for 5 min. 35 cycles each consisting of three steps of denaturation at 94 ˚C for 1 min, annealing at 59 ˚C for 1 min, extension at 72 ˚C for 1 minute and final extension at 72˚C for 5 minutes.The expected 583bp PCR products were electrophoresed on 1.5% agarose gel at presence of 50 bp DNA Ladder (Bioron-Germany) and negative control samples (fig. 1).

Enzyme Digestion

For enzyme digestion, 10µl PCR products, 2µl of 10X Enzyme buffer (B+) and 1µl MboII Enzyme (Fermentas Co.),7µl dH2O were mixed in a tube. The Mixture was transferred to the Thermal Cycler device and incubated at 65˚C for 2 hours and 37˚C for 25 minutes and digested products were electrophoresed on 2% agarose gel at presence of DNA Ladder and negative control samples. In case of the absence of Mediterranean mutation and the enzymatic digestion, 379 bp, 120 bp, 60 bp, 24 bp bands are visible. If there is a mutation, 379bp band is cut and turns to 276bp and 103 bp. (fig. 2). To control the digestion reactions, a number of PCR products were randomly selected for sequencing andwere analyzed by Chromas Lite version 2.33 software (fig. 3).

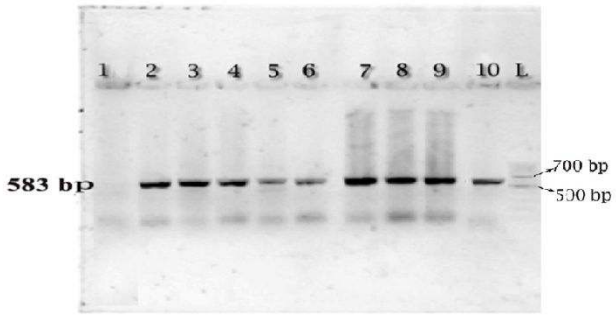

Fig. 1: The expected 583bp PCR products on 1.5% agarosegel. Lanes 2-10: PCR product. Lane 1: negative control. L:50 bp DNA Ladder.

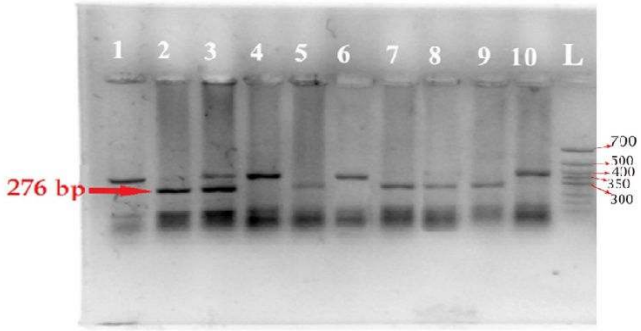

Fig. 2: Restriction enzyme digestion analysis of PCR products related to G6PD Mediterranean mutation with MboII .Lanes 1, 4, 6 ,10 normal samples. Lane2:G6PD-Med Positive control. Lanes:5 ,7 ,8 ,9:G6PDMediterranean mutation. Lane 3: A heterozygote sample.

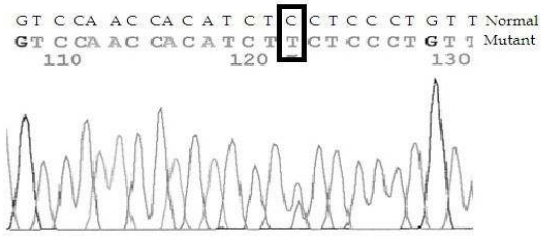

Figure 3. Sequence analysis of exon 6 of G6PD gene. The box indicates C–T nucleotide alteration leading to a change of amino acid Serine to Phenyl Alanine at codon 188 characteristic of Mediterranean mutation.

(TCC 188 TTC) →(Ser 188 Phe)

Results

All of the 90 patients obtained in this study were diagnosed as G6PD deficient by the fluorescent spot test.DNA samples were analyzed for Mediterranean mutation (Ser 188 Phe). G6PD Mediterranean mutation (563 C-T) was observed in 61 (49 males and 12 females) cases, which represents the prevalence rate of 67.77%. 29 patients (32/23%) weren't affected by the Mediterranean-type mutation. Mediterranean defect prevalence rate was 80/3% in men and 19/7% in women and it shows the high incidence of this disease in males and X-Linked recessive pattern of inheritance. Among women, 9 cases (75%) were

heterozygote females and 3 cases (25%) were homozygote.

Discussion

The frequency of G6PD deficiency in the Middle East varies widely, ranging from 1% for Egyptian to 11.5% for some ethnical groups of Iran [8]. Agreeable to the report of WHO, the overall incidence of G6PD deficiency among the Iranian population was 10%-14.9% [9].

The availability of molecular studies by PCR and glucose6phosphatedehy drogenasegene sequencing allows accurate diagnosis and characterization of glucose-6-phosphate dehydrogenase deficiency.In this study, we characterized the molecular defects of the glucose-6-phosphate dehydrogenase gene in deficient patients living in north- west of Iran. We identified variants; G6PD Mediterranean.

Mediterranean mutation is a point mutation in nucleotide No. 563 of G6PD gene and changes Cytosine base to Thymine base (C563T) in exon No. 6 of gene (encoding area). This movement causes phenylalanine amino acid to replace serine amino acid in G6PD protein in place No. 188. Mediterranean mutation is common abnormality and is mainly associated with favism (10, 11).

The highest prevalence of Mediterranean mutation is found in Kermanshah and Khoozestan and other provinces of Iran have also reported high prevalence; whereas the lowest frequency was reported in Kerman (63%) (Table1).

Its prevalence in neighboring countries like Saudi Arabia, Oman, Turkey, India, UAE and Pakistan is 80, 74, 77, 60.4, 55.5 and 76% respectively. Its prevalence is also high in countries on the Mediterranean coast like Spain, Italy and Greece (10, 11,12,13); it can be said that its prevalence in Iran is similar to neighboring countries and9 countries on the Mediterranean coast .

the present study, prevalence rate of the mutation is 67.77% which, compared to the reported mutations, represents that the prevalence rate is similar to mazandaran province. This study and the previous studies, conducted in the provinces, reveal that Mediterranean mutation type is the dominant mutation in these areas it means that in Iran, G6PD dominant mutation is Mediterranean type.

Suggestions for healthcare system and further research

Several molecular studies in Iran have shown that Mediterranean, Chatham and Cosenza mutations are the most common types of G6PD gene mutations in that region [25]. High prevalence of G6PD deficiency in Iran demands higher attention on the part of healthcare system. In this regard, genetic counseling and health education are important to prevent the birth of newborns with this enzyme deficiency, particularly in families with a history of this disease.

This high incidence undoubtedly suggests the importance of planning for early diagnosis and treatment of the patients.

Thus, owing to racial and ethnic variety in different parts of Iran, further research must be carried out on ethnicities, races and regions which haven’t been taken into consideration.

Table 1: prevalence of mediterranean mutation in provinces of Iran[17].

Prevalence	Sample size	Sit of study	Authors	Year of publication
Ghaderigandomani[13]	2011	Khuzestan	231	91.3
Hashemigorji[11]	2009	Fars	34	79.4
Hashemigorji [11]	2009	Esfahan	62	83.9
Mesbannamin [15]	2002	Mazandaran	74	66.21
Mortazavi[16]	2002	Tehran	64	73.4
Mortazavi[18]	2010	Sistan andBluchestan	59	85
Mozafari[19]	2009	Kermanshah	60	91.7
Nooridaloii [21]	2003	Guilan	103	86.4
Nooridaloii [12]	2006	Khorasan	76	66
Nooridaloii [22]	2008	Kerman	64	63
Nooridaloii [22]	2008	Yasd	55	64
Nooridaloii [23]	2009	Esfahan	62	83.87
Nooridaloii [23]	2009	Fars	34	79.46
Nooridaloii [24]	2006	Hormozgan	73	79.45
Nooridaloii [25]	2004	Golestan	71	69
Nooridaloii [27]	2007	Mazandaran	74	66.2
Nooridaloii [28]	2005	Sistan and Bluchestan	92	80.42
Nakhaee[20]	2012	Sistan and Bluchestan	101	84.2
Rahimi[26]	2006	Kermanshah	68	91.2
Kazeminezhad[14]	2009	Khuzestan	144	72.91

Another suggestion for further research can be the fact that Since more than 130 different mutations have been described for the g6pd gene[8], So by using of RFLP-PCR,SSCP and DNA Sequencing techniques and methods the unknown mutations can be investigated. Because the investigation of all of the mutations is time consuming and costs very much, so it suggested investigating the highly prevalent mutations likechatham.

Acknowledgement

We would like to appreciate the respected authorities of the Hematology Laboratory of Koodakan-E-Tabriz (children hospital of

Tabriz), Bu Ali Ardabil and shahid Motahari hospitals to give samples and also we would like to appreciate authorities of the Marand branch Islamic Azad University to allow for the use of equipment and facilities of Genetics Laboratory.

References

1) Mehta A., Mason P., and Valliamy T.J. (2002) ,“ Glocuse-6-phosphate dehydrogenase deficiency,” *Bailliers Clinical Hematology*, **13**: 21-38.

2) Cheng M.L., Ho H.Y., Wu Y.H., and Chiu D.T. (2004) ,“ Glucose-6-phosphate dehydrogenase deficiency cells show increa-sed propensity for oxidant-induced senescence,” *Free Radical Biology and Medicine*, **36**(5): 580-591.

3) Beutler E. (1989) ,“Molecular heterogeneity of glucose-6-phasphate dehydrogenase, *Blood*,” **74**: 2550.

4) Haung Ch.-Sh. (1995) ,“ Neonatal jaundice and molecular mutation in G6PD deficient newborn infants,”American Journal of Hematology, **51**: 19-25.

5) Noori-Daloii MR (2007) ,“ A comprehensive study on the major mutations in G6PD deficient polymorphic variants identified in the coastal provinces of Caspian Sea in the north of Iran,” Clin Biochem., 40: 699-704.

6) Valizadeh M, Onsori H, Fathi A, Bonyadi MJ, Rezamand A, Amani F.(2014),“ Molecular Identification of Mediterranean Mutation in Patients with Deficiency of Glucose-6-Phosphate Dehydrogenase (G6pd) in North West of Iran,” J. Phys. Pharm. Adv.; 4(7): 389-395.

7) Saremi Mohammad Ali et al.(2010) ,“ Rapid genomic DNA extraction (RGDE) ,”Protocol Online PID: 4791, http://www.protocol-online.org

8) Noori-Daloii MR, Soltanian S, Mohammad Gangi S, Yousefi A, Hejazi S, Bani- Hashem A, Hitratfar S, Sanati MH (2006) ,“ Molecular identification of the most prevalent mutations of g6pd gene in deficient patients in Khorasan province of Iran,” *J Sci R Iran*, 17(2): 103-106.

9) WHO Working Group (1989) ,“ World map of G6PD deficincy,” *Bull WHO*, 67: 601-611.

10) Farhud DD, Yazdanpanah L(2008) ,“ Glucose-6- phosphate dehydrogenase (G6PD) Deficiency,”*Iranian J Publ Health, 37(4):1-18*.

11) Hashemi Gorji F, Alivand M, Atef Vahid P, Hashemi M, Salehi R, Salehi M, et al.(2009) ,“ Study of glucose-6-phosphate dehydrogenese (G6PD) gene mutations in patients with G6PD deficiency of Fars and Esfahan provinces,” *J Islamic Azad university,* 19(4):280-286.

12) Noori-Daloii MR, Soltanian S, Mohammad Gangi SH, Yousefi A, Hejazi S, Banihashem A, et al.(2006) ,“ Molecular Identification of the Most Prevalent Mutations of Glucose-6-Phosphate Dehydrogenase (G6PD) Gene in deficient Patients in Khorasan Province of Iran,” *J Sci I R Iran*, 17(2):103-106.

13) Gandomani MG, Khatami SR, Kazeminezhad SR, Daneshmand S, Mashayekhi A.(2011) ,“ Molecular Identification of G6PD Chatham (G1003A)in Khuzestan Province of Iran,” *Journal of Genetics*, 90(1):143-145.

14) *Kazemi Nezhad* SR, *Mashayekhi* A, *Khatami* SR, *Daneshmand* S, *Fahmi* F, *Ghaderigandmani* M, et al. (2009) ,“ Prevalence and Molecular Identification of Mediterranean Glucose-6-Phosphate Dehydrogenase Deficiency in Khuzestan Province, Iran ,” *Iranian J Publ Health, 38(3):127.*

15) Mesbah Namin SA, Sanati MH, Mowjoodi A, Mason PJ, Vulliamy TJ, Noori Daloii MR(2002) ,“ Three major glucose-6-phosphate dehydrogenase–deficient

polymorphic variants identified in Mazandaran state of Iran," *Br J Haematol,* 117(3):763-4.

16) Mortazavi Y, Tarmahiardastani M, Poorfathollah AA(2002) ," The survey of mutation Molecular of Mediterranean in people of Glucose-6- Phosphate Dehydrogenase Deficiency in Tehran 2001-2002,"*J of Zanjan university of medical science,* 38:26-31.

17) Moosazadeh M, Nekoei-Moghadam M, Aliramzany M, Amiresmaili M.(2013) ,"Identification of Mutation of Glucose-6-Phosphate Dehydrogenase (G6PD) in Iran: Meta- analysis Study," *Iranian J Publ Health,42(9):* 1007-1015.

18) Mortazavi Y, Mirzamohammadi F, Teremahi Ardestani M, Mirimoghadam E, Vulliamy TJ(2010) ," Glucose 6-phosphate dehydrogenase deficiency in Tehran, Zanjan and Sistan- Balouchestan provinces: prevalence and frequency of Mediterranean variant of G6PD," *Iran J Biotechnol*, 8(4):229-233.

19) Mozafari H, Rahimi Z, Heidarpour A, Fallahi M, Muniz A(2009) ," The prevalence of factor V Leiden, prothrombin G20210A and methylenetetrahydrofolate reductase polymorphism C677T among G6PD deficient individuals from Western Iran ,"*Mol Biol Rep*, 36:2361–2364.

20) Nakhaee A, Salimi S, Zadehvakili A, Dabiri S, Noora M, Rezaei M, et al. (2012) ," The Prevalence of Mediterranean Mutation of Glucose- 6-Phosphate Dehydrogenase (G6PD) in Zahedan,"*Zahedan J Res Med Sci*, 14(3):39-43.

21) Noori-Daloii MR, Hajebrahimi Z, Najafi L(2003) ," Molecular identification of the most prevalent mutation of glucose-6-phosphate dehydrogenase gene in deficient patients in Gilan province,"*J Sci IR Iran*, 14(4): 327-31.

22) Noori-Daloii MR, Alivand MR, Mir-Arabshahi P, Yekaninejad MS(2008) ," Molecular Surveying of the Common Variants of Glucose 6-Phosphate Dehydrogenase Gene in Deficient Patients in Two Neighboring Provinces, Kerman and Yazd in Central Part of Iran," *J Sci I R Iran*, 19(4): 317-322.

23) Noori-Daloii MR, Hashemi-Gorji F, Alivand MR, Hashemi R, Mirfakharaie R, Atef-vahid M, et al(2009) ," Molecular Identification of the Most Prevalent Mutations of Glucose-6-Phosphate Dehydrogenase (G6PD) in Fars and Isfahan of Iran,"*J Sci I R Iran*, 20(3): 221-225.

24) Noori-Daloii MR, Hejazi, SH, Yousefi A, Mohammad ganji S, Soltani S, Javadi KR, et al(2006) ," Identification of Mutations in G6PD Gene in Patients in Hormozgan Province of Iran,"*J Sci I R Iran*, 17(4): 313-316.

25) Noori Daloii MR, Najafi L, Mohammad Ganji S, Hajebrahimi Z, Sanati MH(2004) ," Molecular identification of mutations in G6PD gene in patients with favism in Iran,"*J Physiol Biochem*, 60(4); 273-7.

26) Rahimi Z, Vaisi-Raygani A, Nagel RL, et al.(2006) ," Molecular characterization of glucose-6-phosphate dehydrogenase deficiency in the Kurdish population of Western Iran," *Blood Cells Mol Dis*, 37:91–4.

27) Noori-Daloii MR, Hajebrahimi Z, Najafi L(2007) ," A comprehensive study on the major mutations in glucose-6-phosphate dehydrogenasedeficient polymorphic variants identified in the coastal provinces of Caspian Sea in the north of Iran," *Clin Biochem*, 40(9-10):699–704.

24. Noori-Daloii MR, Yousefi A, Mohammad Ganji S, Hejazi S, Soltanian S, Sanei Moghadam E, Bozorgzade P, Sanati MH(2005) ,"Molecular identification of the most prevalent mutations of g6pd gene in deficient patients in Sistan and Baluchestan province of Iran," *J Sci IR Iran*, 16(4): 321-25.

مطالعه مولکولی گلوکز –۶ فسفات دهیدروژناز مدیترانه ای در شمالغرب ایران

افشین فتحی [۱]، مهدی ولی زاده [۲]،حبیب عنصری [۳]، روح اله مرادپور[٤]، فیروز امانی [٥]

۱- استادیار گروه هماتولوژی و انکولوژی کودکان، دانشگاه علوم پزشکی اردبیل

۲- گروه ژنتیک، کارشناس ارشد ژنتیک

۳- استادیار گروه زیست شناسی سلولی و مولکولی، دانشگاه آزاد اسلامی واحد مرند

٤- گروه ژنتیک، کارشناس ارشد ژنتیک

٥- استادیار گروه آمار زیستی، دانشگاه علوم پزشکی اردبیل

نویسنده مسئول: استادیار گروه هماتولوژی و انکولوژی کودکان، دانشگاه علوم پزشکی اردبیل ، اردبیل، ایران

چکیده

نقص آنزیم گلوکز٦- فسفات دهیدروژناز شایعترین نقص آنزیمی انسانی است که بیش از ٤۰۰ میلیون نفردر سراسر جهان به آن مبتلا هستند این آنزیم مرحله اول در مسیر پنتوز فسفات را کاتالیز می کند که در این مرحله گلوکز ٦-فسفات را به ٦-فسفو گلوکونات تبدیل می کند و همراه با آن احیاء $NADP^+$ را منجر می شود . این مسیر یک منبع مهم تولید NADPH است . NADPH، با حفظ گلوتاتیون به شکل احیاء در حفاظت سلول در برابر تنش های اکسیداتیو نقش دارد.

هدف این مطالعه شناسایی مولکولی جهش مدیترانه ای گلوکز-٦-فسفات دهیدروژنازدربیماران مبتلا درشمالغرب ایران بود. درمطالعه حاضراز ۹۰ نمونه خون بیماران زن و مرد غیر خویشاوند DNA, توسط روش استخراج DNA ژنومی سریع (RGDE) استخراج شد. به منظورجستجوی جهش مدیترانه ای، روش های تعیین توالی وPCR-RFLP مورداستفاده قرارگرفتند. این مطالعه نشان داد که ٦۱ نمونه از ۹۰ نمونه (67.77%) جهش مدیترانه ای دارند. داده ها نشان میدهند که جهش G6PD مدیترانه ای شایعترین نوع جهش درشمالغرب ایران است.

واژهای کلیدی: گلوکز ٦-فسفات دهیدروژناز، جهش مدیترانه ای ، شمالغرب ایران

Molecular investigation of Mediterranean glucose-6-phosphate dehydrogenase in North West of Iran

Fathi Afshin[1],Mehdi Valizadeh[2],Onsori Habib[3],Moradpour Rouhollah[4],Amani Firouz[5].

1- Pediatric Hematology & Oncology Department. Ardabil University of Medical Sciences, Ardabil, Iran
2- Genetics Department, M. A. in Genetics
3- Cellular and Molecular Biology Department,Marand Branch, Islamic Azad University, Marand, Iran
4-Genetics Department, M .A. in Genetics
5- Community Medicine Department, Ardabil University of Medical Science, Ardabil, Iran

Corresponding author: Pediatric Hematology & Oncology Department. Ardabil University of Medical Sciences, Ardabil, Iran

Abstract

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common human enzyme deficiency affecting more than 400 million people worldwide.This enzyme catalyse the first step in pentose phosphate pathway (conversion of glucose-6-phosphate to 6-phosphogluconat) with the concomitant reduction of $NADP^+$. This pathway is an important source of NADPH. By preserving and regenerating reduced form of glutathione, NADPH plays a major role in a cell's stability to withstand oxidative stress. The aim of this study was molecular identification of Mediterranean mutation in Glucose-6-phosphate dehydrogenase in affected patients in North West of Iran. In the present study,from 90 blood samples of unrelated male and female patients, DNA was extracted by Rapid Genomic DNA Extraction (RGDE) method.In order to search for Mediterranean mutation, PCR-RFLP and sequencing methods were used.This study, revealed that 61 samples out of 90 have the Mediterranean mutation (67.77%).the data indicate that the G6PD Mediterranean mutation is the most common in North West of Iran.

Keyword: G6PD,Mediterranean, mutation, North West of Iran.

Introduction

The housekeeping enzyme glucose-6-phosphate dehydrogenase (G6PD) catalyzes the first step in pentose phosphate pathway (conversion of glucose-6-phosphat to 6-phosphoglucono lactone) with the concomitant reduction of $NADP^+$. This pathway is an important source of NADPH that is essential for maintaining adequate intracellular level of reduced forms of glutathione and other sulphydryl groups. By preserving and regenerating reduced forms of glutathione as well as promoting the stability of catalase, NADPH plays a major role in the stability of cell to withstand oxidative stress. In red blood cells, since G6PD is the only source of NADPH, defense against oxidative damage is dependent on its activity [1-2]. The G6PD gene is located on the Xq28 region of X chromosome. It contains 13 exons and 12 introns and is 18.5 kb in length. The active enzyme is composed variably of two or four identical 515 amino acid subunits; each monomer has a molecular weight of 59 kDa. G6PD deficiency is the most common human enzymopathy and affects 400 million people worldwide. Although the majority of people with this disease are asymptomatic, some of the clinical symptoms associated with deficiency are acute hemolytic anemia in association with infection or following the ingestion of some drugs or fava beans (favism), neonatal jaundice and in severe deficiency, chronic non-spherocytic hemolytic anemia (CNSHA) [3-4].

Although Iranian population consists of different racial groups, but the overall incidence of G6PD deficiency in Iranian population is estimated around 10%-14.9% [5-6].

According to previous studies, the most prevalent mutations in Iran are Mediterranean, Chatham and Cosenza[6]. The aim of the present study is to investigate the frequency rate of the Mediterranean mutation at 563nt(C→T) in north-west of Iran.

Methodology

In this cross-sectional study,90 Peripheral blood samples(2-5 ml in 300 µEDTA 0.5 M) from unrelated patients with favism were collected from the hospitals of the north-west of Iran (including Ardebil, Tabriz and Urmia provinces). The blood samples collection process was performed with the ethics committee approval and the patients' informed consent through telephone correspondences. The falcons were held in the temperature of -20^{oC}until the DNA extraction. Qualitative measurement of the enzyme activity was performed using Fluorescent Spot Test and Saba laboratory kit. The basis of this method is the catalytic activity of G6PD enzyme in conversion of G6PD Glucose 6-phosphate to 6-Phosphogloconate and simultaneous revival of NADP to NADPH2. The produced NADPH2 has fluorescent specifications under UV (365nm). The fluorescence intensity in the blood of healthy individuals is positive (strong) and in the blood of patients with G6PD deficiency is low or negative.

DNA Extraction and Amplification

Genomic DNA was extracted from Peripheral blood leukocytes using Rapid Genomic DNA Extraction (RGDE) method [7].

All 90 DNA samples were analysed by PCR-RFLP method for the C to T mutation at nt 563, which is characteristic of G6PD Mediterranean.PCR reaction mixture containing 30 pmol of eachprimers (F-Med: 5' CCC CGA AGA GGA ATT CAA GGG GGT 3') as a forward and R-Med: 5' GAA GAG TAG CCC TCG AGG GTG ACT 3') as a reverse, 12/5µl DFS-Taqmaster mix (Bioron-Germany) and about 1µg genomic DNA on a thermal cycler (SensoQuest-Germany).PCR conditions were as follows: Initial denaturation of DNA at 95 ˚C for 5 min. 35 cycles each consisting of three steps of denaturation at 94 ˚C for 1 min, annealing at 59 ˚C for 1 min, extension at 72 ˚C for 1 minute and final extension at 72˚C for 5 minutes.The expected 583bp PCR products were electrophoresed on 1.5% agarose gel at presence of

50 bp DNA Ladder (Bioron-Germany) and negative control samples (fig. 1).

Enzyme Digestion

For enzyme digestion, 10µl PCR products, 2µl of 10X Enzyme buffer (B+) and 1µl MboII Enzyme (Fermentas Co.),7µl dH2O were mixed in a tube. The Mixture was transferred to the Thermal Cycler device and incubated at 65˚C for 2 hours and 37˚C for 25 minutes and digested products were electrophoresed on 2% agarose gel at presence of DNA Ladder and negative control samples. In case of the absence of Mediterranean mutation and the enzymatic digestion, 379 bp, 120 bp, 60 bp, 24 bp bands are visible. If there is a mutation, 379bp band is cut and turns to 276bp and 103 bp. (fig. 2). To control the digestion reactions, a number of PCR products were randomly selected for sequencing andwere analyzed by Chromas Lite version 2.33 software (fig. 3).

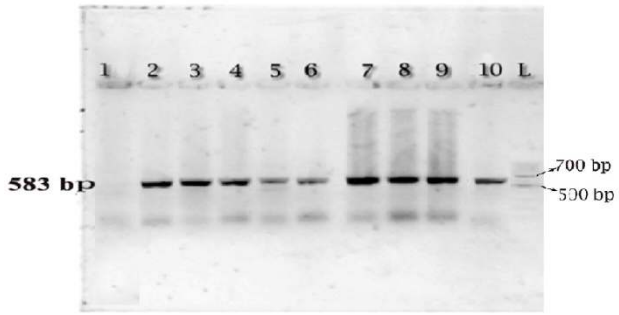

Fig. 1: The expected 583bp PCR products on 1.5% agarosegel. Lanes 2-10: PCR product. Lane 1: negative control. L:50 bp DNA Ladder.

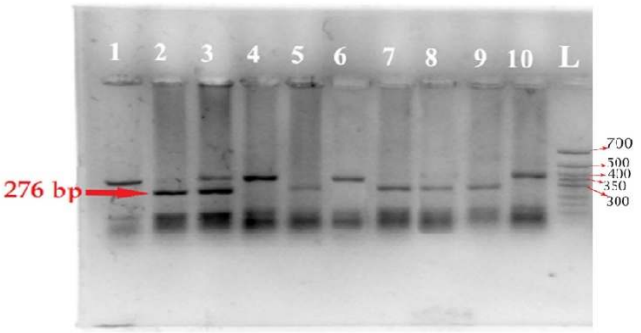

Fig. 2: Restriction enzyme digestion analysis of PCR products related to G6PD Mediterranean mutation with MboII .Lanes 1, 4, 6 ,10 normal samples. Lane2:G6PD-Med Positive control. Lanes:5 ,7 ,8 ,9:G6PDMediterranean mutation. Lane 3: A heterozygote sample.

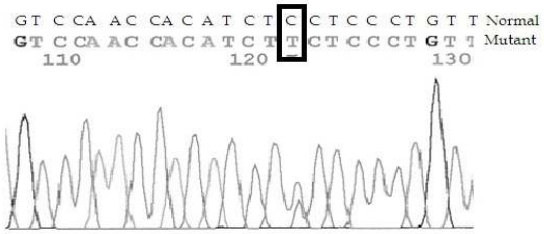

Figure 3. Sequence analysis of exon 6 of G6PD gene. The box indicates C–T nucleotide alteration leading to a change of amino acid Serine to Phenyl Alanine at codon 188 characteristic of Mediterranean mutation. (TCC 188 TTC) →(Ser 188 Phe)

Results

All of the 90 patients obtained in this study were diagnosed as G6PD deficient by the fluorescent spot test.DNA samples were analyzed for Mediterranean mutation (Ser 188 Phe). G6PD Mediterranean mutation (563 C-T) was observed in 61 (49 males and 12 females) cases, which represents the prevalence rate of 67.77%. 29 patients (32/23%) weren't affected by the Mediterranean-type mutation. Mediterranean defect prevalence rate was 80/3% in men and 19/7% in women and it shows the high incidence of this disease in males and X-Linked recessive pattern of inheritance. Among women, 9 cases (75%) were heterozygote females and 3 cases (25%) were homozygote.

Discussion

The frequency of G6PD deficiency in the Middle East varies widely, ranging from 1% for Egyptian to 11.5% for some ethnical groups of Iran [8]. Agreeable to the report of WHO, the overall incidence of G6PD deficiency among the Iranian population was 10%-14.9% [9].

The availability of molecular studies by PCR and glucose6phosphatedehy drogenasegene sequencing allows accurate diagnosis and characterization of glucose-6-phosphate dehydrogenase deficiency.In this study, we characterized the molecular defects of the glucose-6-phosphate dehydrogenase gene in deficient patients living in north- west of Iran. We identified variants; G6PD Mediterranean. Mediterranean mutation is a point mutation in nucleotide No. 563 of G6PD gene and changes Cytosine base to Thymine base (C563T) in exon No. 6 of gene (encoding area). This movement causes phenylalanine amino acid to replace serine amino acid in G6PD protein in place No. 188. Mediterranean mutation is common abnormality and is mainly associated with favism (10, 11). The highest prevalence of Mediterranean mutation is found in Kermanshah and Khoozestan and other provinces of Iran have also reported high

prevalence; whereas the lowest frequency was reported in Kerman (63%) (Table1).
Its prevalence in neighboring countries like Saudi Arabia, Oman, Turkey, India, UAE and Pakistan is 80, 74, 77, 60.4, 55.5 and 76% respectively. Its prevalence is also high in countries on the Mediterranean coast like Spain, Italy and Greece (10, 11,12,13); it can be said that its prevalence in Iran is similar to neighboring countries and9 countries on the Mediterranean coast .
the present study, prevalence rate of the mutation is 67.77% which, compared to the reported mutations, represents that the prevalence rate is similar to mazandaran province. This study and the previous studies, conducted in the provinces, reveal that Mediterranean mutation type is the dominant mutation in these areas it means that in Iran, G6PD dominant mutation is Mediterranean type.

Suggestions for healthcare system and further research

Several molecular studies in Iran have shown that Mediterranean, Chatham and Cosenza mutations are the most common types of G6PD gene mutations in that region [25]. High prevalence of G6PD deficiency in Iran demands higher attention on the part of healthcare system. In this regard, genetic counseling and health education are important to prevent the birth of newborns with this enzyme deficiency, particularly in families with a history of this disease.
This high incidence undoubtedly suggests the importance of planning for early diagnosis and treatment of the patients.
Thus, owing to racial and ethnic variety in different parts of Iran, further research must be carried out on ethnicities, races and regions which haven't been taken into consideration.

Table 1: prevalence of mediterranean mutation in provinces of Iran[17].

Authors	Year of publication	Sit of study	Sample size	Prevalence
Ghaderigandomani[13]	2011	Khuzestan	231	91.3
Hashemigorji[11]	2009	Fars	34	79.4
Hashemigorji [11]	2009	Esfahan	62	83.9
Mesbannamin [15]	2002	Mazandaran	74	66.21
Mortazavi[16]	2002	Tehran	64	73.4
Mortazavi[18]	2010	Sistan andBluchestan	59	85
Mozafari[19]	2009	Kermanshah	60	91.7
Nooridaloii [21]	2003	Guilan	103	86.4
Nooridaloii [12]	2006	Khorasan	76	66
Nooridaloii [22]	2008	Kerman	64	63
Nooridaloii [22]	2008	Yasd	55	64
Nooridaloii [23]	2009	Esfahan	62	83.87
Nooridaloii [23]	2009	Fars	34	79.46
Nooridaloii [24]	2006	Hormozgan	73	79.45
Nooridaloii [25]	2004	Golestan	71	69
Nooridaloii [27]	2007	Mazandaran	74	66.2
Nooridaloii [28]	2005	Sistan and Bluchestan	92	80.42
Nakhaee[20]	2012	Sistan and Bluchestan	101	84.2
Rahimi[26]	2006	Kermanshah	68	91.2
Kazeminezhad[14]	2009	Khuzestan	144	72.91

Another suggestion for further research can be the fact that Since more than 130 different mutations have been described for the g6pd gene[8], So by using of RFLP-PCR,SSCP and DNA Sequencing techniques and methods the unknown mutations can be investigated. Because the investigation of all of the mutations is time consuming and costs very much, so it suggested investigating the highly prevalent mutations likechatham.

Acknowledgement

We would like to appreciate the respected authorities of the Hematology Laboratory of Koodakan-E-Tabriz (children hospital of Tabriz), Bu Ali Ardabil and shahid Motahari hospitals to give samples and also we would like to appreciate authorities of the Marand branch Islamic Azad University to allow for the use of equipment and facilities of Genetics Laboratory.

References

1) Mehta A., Mason P., and Valliamy T.J. (2002) ,“ Glocuse-6-phosphate dehydrogenase deficiency,” *Bailliers Clinical Hematology*, **13**: 21-38.

2) Cheng M.L., Ho H.Y., Wu Y.H., and Chiu D.T. (2004) ,“ Glucose-6-phosphate dehydrogenase deficiency cells show increa-sed propensity for oxidant-induced senescence,” *Free Radical Biology and Medicine*, **36**(5): 580-591.

3) Beutler E. (1989) ,“Molecular heterogeneity of glucose-6-phasphate dehydrogenase, *Blood*,” **74**: 2550.

4) Haung Ch.-Sh. (1995) ,“ Neonatal jaundice and molecular mutation in G6PD deficient newborn infants,”American Journal of Hematology, **51**: 19-25.

5) Noori-Daloii MR (2007) ,“ A comprehensive study on the major mutations in G6PD deficient polymorphic variants identified in the coastal provinces of Caspian Sea in the north of Iran,” Clin Biochem., 40: 699-704.

6) Valizadeh M, Onsori H, Fathi A, Bonyadi MJ, Rezamand A, Amani F.(2014),“ Molecular Identification of Mediterranean Mutation in Patients with Deficiency of Glucose-6-Phosphate Dehydrogenase (G6pd) in North West of Iran,” J. Phys. Pharm. Adv.; 4(7): 389-395.

7) Saremi Mohammad Ali et al.(2010) ,“ Rapid genomic DNA extraction (RGDE) ,”Protocol Online PID: 4791, http://www.protocol-online.org

8) Noori-Daloii MR, Soltanian S, Mohammad Gangi S, Yousefi A, Hejazi S, Bani- Hashem A, Hitratfar S, Sanati MH (2006) ,“ Molecular identification of the most prevalent mutations of g6pd gene in deficient patients in Khorasan province of Iran,” *J Sci R Iran*, 17(2): 103-106.

9) WHO Working Group (1989) ,“ World map of G6PD deficincy,” *Bull WHO*, 67: 601-611.

10) Farhud DD, Yazdanpanah L(2008) ,“ Glucose-6- phosphate dehydrogenase (G6PD) Deficiency,”*Iranian J Publ Health, 37(4):1-18*.

11) Hashemi Gorji F, Alivand M, Atef Vahid P, Hashemi M, Salehi R, Salehi M, et al.(2009) ,“ Study of glucose-6-phosphate dehydrogenese (G6PD) gene mutations in patients with G6PD deficiency of Fars and Esfahan provinces,” *J Islamic Azad university,* 19(4):280-286.

12) Noori-Daloii MR, Soltanian S, Mohammad Gangi SH, Yousefi A, Hejazi S, Banihashem A, et al.(2006) ,“ Molecular Identification of the Most Prevalent Mutations of Glucose-6-Phosphate Dehydrogenase (G6PD) Gene in deficient Patients in Khorasan Province of Iran,” *J Sci I R Iran*, 17(2):103-106.

13) Gandomani MG, Khatami SR, Kazeminezhad SR, Daneshmand S, Mashayekhi A.(2011) ,“ Molecular Identification of G6PD Chatham (G1003A)in Khuzestan Province of Iran,” *Journal of Genetics*, 90(1):143-145.

14) *Kazemi Nezhad* SR, *Mashayekhi* A, *Khatami* SR, *Daneshmand* S, *Fahmi* F, *Ghaderigandmani* M, et al. (2009) ,“ Prevalence and Molecular Identification of Mediterranean Glucose-6-Phosphate Dehydrogenase Deficiency in Khuzestan Province, Iran ,” *Iranian J Publ Health, 38(3):127.*

15) Mesbah Namin SA, Sanati MH, Mowjoodi A, Mason PJ, Vulliamy TJ, Noori Daloii MR(2002) ,“ Three major glucose-6-phosphate dehydrogenase–deficient polymorphic variants identified in Mazandaran state of Iran,” *Br J Haematol,* 117(3):763-4.

16) Mortazavi Y, Tarmahiardastani M, Poorfathollah AA(2002) ,“ The survey of mutation Molecular of Mediterranean in people of Glucose-6- Phosphate Dehydrogenase Deficiency in Tehran 2001-2002,”*J of Zanjan university of medical science,* 38:26-31.

17) Moosazadeh M, Nekoei-Moghadam M, Aliramzany M, Amiresmaili M.(2013) ,“Identification of Mutation of Glucose-6-Phosphate Dehydrogenase (G6PD) in Iran: Meta- analysis Study,” *Iranian J Publ Health,42(9):* 1007-1015.

18) Mortazavi Y, Mirzamohammadi F, Teremahi Ardestani M, Mirimoghadam E, Vulliamy TJ(2010) ,“ Glucose 6-phosphate dehydrogenase deficiency in Tehran, Zanjan and Sistan- Balouchestan provinces: prevalence and frequency of Mediterranean variant of G6PD,” *Iran J Biotechnol*, 8(4):229-233.

19) Mozafari H, Rahimi Z, Heidarpour A, Fallahi M, Muniz A(2009) ,“ The prevalence of factor V Leiden, prothrombin G20210A and methylenetetrahydrofolate reductase polymorphism C677T among G6PD deficient individuals from Western Iran ,”*Mol Biol Rep*, 36:2361–2364.

20) Nakhaee A, Salimi S, Zadehvakili A, Dabiri S, Noora M, Rezaei M, et al. (2012) ,“ The Prevalence of Mediterranean Mutation of Glucose- 6-Phosphate Dehydrogenase (G6PD) in Zahedan,”*Zahedan J Res Med Sci*, 14(3):39-43.

21) Noori-Daloii MR, Hajebrahimi Z, Najafi L(2003) ,“ Molecular identification of the most prevalent mutation of glucose-6-phosphate dehydrogenase gene in deficient patients in Gilan province,”*J Sci IR Iran*, 14(4): 327-31.

22) Noori-Daloii MR, Alivand MR, Mir-Arabshahi P, Yekaninejad MS(2008) ,“ Molecular Surveying of the Common Variants of Glucose 6-Phosphate Dehydrogenase Gene in Deficient Patients in Two Neighboring Provinces, Kerman and Yazd in Central Part of Iran,” *J Sci I R Iran*, 19(4): 317-322.

23) Noori-Daloii MR, Hashemi-Gorji F, Alivand MR, Hashemi R, Mirfakharaie R, Atef-vahid M, et al(2009) ,“ Molecular Identification of the Most Prevalent Mutations of Glucose-6-Phosphate Dehydrogenase (G6PD) in Fars and Isfahan of Iran,”*J Sci I R Iran*, 20(3): 221-225.

24) Noori-Daloii MR, Hejazi, SH, Yousefi A, Mohammad ganji S, Soltani S, Javadi KR, et al(2006) ,“ Identification of Mutations in G6PD Gene in Patients in Hormozgan Province of Iran,”*J Sci I R Iran*, 17(4): 313-316.

25) Noori Daloii MR, Najafi L, Mohammad Ganji S, Hajebrahimi Z, Sanati MH(2004) ,“ Molecular identification of mutations in G6PD gene in patients with favism in Iran,”*J Physiol Biochem*, 60(4); 273-7.

26) Rahimi Z, Vaisi-Raygani A, Nagel RL, et al.(2006) ,“ Molecular characterization of glucose-6-phosphate dehydrogenase deficiency in the Kurdish population of Western Iran,” *Blood Cells Mol Dis*, 37:91–4.

27) Noori-Daloii MR, Hajebrahimi Z, Najafi L(2007) ,“ A comprehensive study on the major mutations in glucose-6-phosphate dehydrogenasedeficient polymorphic variants identified in the coastal provinces of Caspian Sea in the north of Iran,” *Clin Biochem*, 40(9-10):699–704.

24. Noori-Daloii MR, Yousefi A, Mohammad Ganji S, Hejazi S, Soltanian S, Sanei Moghadam E, Bozorgzade P, Sanati MH(2005) ,“Molecular identification of the most prevalent mutations of g6pd gene in deficient patients in Sistan and Baluchestan province of Iran,” *J Sci IR Iran*, 16(4): 321-25.